(ABI 377; PE Biosystems) was Ampli-Taq DNA polymerase. Another problem may be attributable to the production of uneven peak-height patterns, which are caused by differences in the efficiency of dideoxy termination at different bases and are affected by sequence context as well. Zakeri et al. *(5)* reported that a heterozygous C peak was much smaller in size than a heterozygous G peak. This is in agreement with our results. In addition to the production of uneven peaks, a high background, produced by impurities in the DNA template, could further complicate the results, especially for heterozygote detection.

In our case, the background in the electropherograms was fairly low because all the 998-bp DNA fragments were purified by gel extraction. The quality of sequence data was actually very good for each sample, allowing us to read a sequence of ~500 nucleotides, which matched completely with the published *NAT2* gene sequence *(9)*. However, in a few samples, the signals produced by heterozygosity at the $T^{341}C$ and $C^{282}T$ sites were below the set default value (30%) for heterozygote detection. For example, our quality-control sample (sample 20) could be scored as a borderline heterozygote in the reverse sequencing reaction (27% G and 100% A) but not with the forward primer. If the threshold value of heterozygote detection was set lower, e.g., 20%, then sample 10 could also be scored as a heterozygote, at least in one sequencing direction. When a scoring problem arises for a sample after sequencing in both directions, it should be considered an undefined genotype until an independent method is used for its identification. We therefore used independent PCR-RFLP methods to check the genotypes of all 20 samples at three different polymorphic sites in the *NAT2* gene, and discrepancies were found between the sequencing and RFLP methods for only four test samples (Table 1, samples 8, 9, 10, and 11).

In conclusion, there are some inherent problems in automated DNA sequencing, which may lead to inaccurate heterozygote identification in some samples. Until the associated problems are fully resolved, precautions should be taken in the use of automated sequencing for heterozygote detection. If possible, we recommend the use of two complementary PCR-RFLP methods in this type of analysis.

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Increased Maternal Plasma Fetal DNA Concentrations in Women Who Eventually Develop Preeclampsia, *Tse N. Leung,[1] Jun Zhang,[2] Tze K. Lau,[1] Lisa Y.S. Chan,[2] and Y.M. Dennis Lo[2*]* (Departments of [1] Obstetrics and Gynecology and [2] Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong SAR; [*] address correspondence to this author at: Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Room 38023, 1/F Clinical Sciences Bldg., 30-32 Ngan Shing St., Shatin, New Territories, Hong Kong SAR; fax 852-2194-6171, e-mail loym@cuhk.edu.hk)

Preeclampsia is a multisystem disorder specific to pregnant women. It remains one of the most important causes of maternal and fetal mortality and morbidity in developed countries *(1)*. Although the pathogenesis of this condition is not fully understood, it is now widely accepted that vascular endothelial cell dysfunction is the final common pathway responsible for the maternal syndrome *(2, 3)*. The underlying pathological changes that lead to the endothelial cell dysfunction remain incompletely understood, but poor placentation has been proposed as a major contributory factor *(2, 4, 5)*. As a result of incomplete or failure of trophoblastic invasion of the spiral arteries, placental ischemia results, leading to the release of one or more factors that are responsible for the damage of the maternal vascular endothelium *(5, 6)*. The normal process of trophoblastic invasion is complete by 20 weeks of gestation. Hence, the initiating placental pathology should exist prior to this stage of pregnancy, long before the onset of the clinical syndrome. Therefore, it might be possible to develop new plasma/serum biochemical markers for identifying subjects at increased risk of developing preeclampsia.

We previously have shown that women with established preeclampsia have a fivefold increase in circulating fetal DNA concentrations in their plasma compared with control pregnant subjects *(7)*. However, it is unknown whether this is a late phenomenon or whether it precedes the onset of clinical symptoms and signs. In this study, we aimed to test whether the abnormal increase in circulating fetal DNA concentrations can be detected in susceptible subjects before onset of the clinical disease.

Pregnant women attending the Department of Obstetrics and Gynecology, Prince of Wales Hospital, Hong Kong were recruited with informed consent. The study was approved by the Clinical Research Ethics Committee

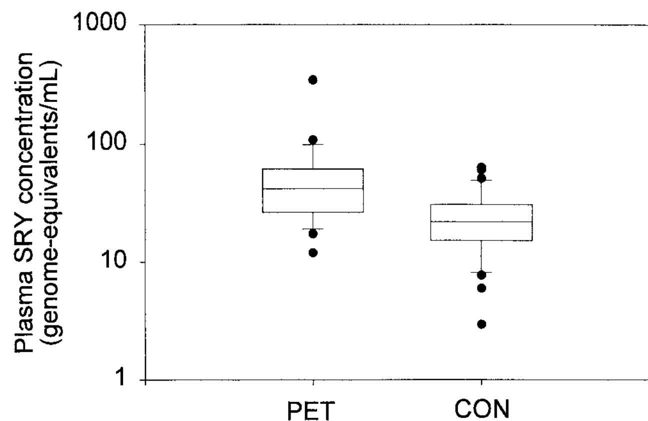

Fig. 1. Mid-trimester maternal plasma fetal DNA concentrations in preeclamptic (*PET*; n = 18) and control (*CON*; n = 33) subjects.

The *y-axis* is in the common logarithmic scale. The *lines* inside the *boxes* denote the medians. The *boxes* mark the interval between the 25th and 75th percentiles. The *whiskers* denote the interval between the 10th and 90th percentiles. ● indicates outliers outside the 5th and 95th percentiles.

of The Chinese University of Hong Kong. Only singleton pregnancies were included. The gestational ages of all studied subjects were confirmed by early ultrasound examination. Antecubital venous blood (10 mL) was collected from each subject between 11 and 22 weeks of gestation and placed immediately into EDTA tubes. After centrifugation at 3000*g* for 20 min, the plasma samples were collected into plain polypropylene tubes and stored at −70 °C until further processing. All subjects were followed until delivery, and all relevant clinical information was recorded.

Of those who carried male fetuses and had blood sampled, 18 subjects subsequently developed preeclampsia as the only antenatal complication. The gestational age at onset of clinical disease was 27.4–40.0 weeks. Preeclampsia was defined essentially as described previously *(8)*, on the basis of a diastolic blood pressure >110 mmHg on one occasion or >90 mmHg on two or more occasions at least 4 h apart, with the presence of significant proteinuria in subjects with no history of hypertension. Significant proteinuria was defined as proteinuria >0.3 g/day or ≥2+ on dipstick testing in two clean-catch midstream urine specimens collected at least 4 h apart. Another 33 subjects who carried male fetuses and had no antenatal complication were randomly selected as the control group. The mean gestational ages of the preeclamptic and control subjects were 17.2 weeks (SD, 2.9 weeks) and 18.0 weeks (SD, 1.3 weeks), respectively. There was no statistically significant difference in the gestational ages at blood sampling between the preeclamptic and control groups (P = 0.23).

Plasma samples from these two groups were assayed for circulating fetal DNA, using the *SRY* gene on the Y chromosome as a marker, as described previously *(7)*. The sensitivity and precision of this assay have been reported previously *(7)*. As a control for the amplifiability of plasma-extracted DNA, all samples were subjected to a TaqMan assay for the *β-globin* gene on chromosome 11. Samples from 10 pregnant subjects carrying female fetuses during the second trimester were also assayed as negative controls. The laboratory staff responsible for the molecular analysis of the samples were unaware of the clinical status of the subjects from whom the samples were obtained.

Circulating fetal DNA was detected in all subjects carrying male fetuses in both the preeclamptic and control groups. Fig. 1 shows the plasma fetal DNA concentrations of these two groups of subjects. The median fetal DNA concentrations in preeclamptic and control pregnancies were 41.9 genome-equivalents/mL (interquartile range, 25.8–62.8; range, 36.3–2375) and 22.0 genome-equivalents/mL (interquartile range, 15.3–31.5; range, 4.25–300), respectively. Fetal DNA concentrations were significantly higher in preeclamptic than control pregnancies (Mann–Whitney rank-sum test, P = 0.001; U-statistic = 340.5). None of the plasma samples from the 10 women carrying female fetuses had any *SRY* signal.

Positive amplification signals from the *β-globin* gene were detected in all tested samples, thus confirming the quality of the DNA samples. There was no significant difference in the plasma concentrations of the *β-globin* gene between the preeclamptic and control groups (Mann–Whitney rank-sum test, P = 0.16).

Our data indicate that increased concentrations of maternal plasma fetal DNA could be detected in susceptible subjects before the onset of clinical presentation of preeclampsia. The mechanisms producing this increase require further investigation. Previously, we proposed that the possible pathways for maternal plasma fetal DNA increase after the onset of preeclampsia include increased liberation of fetal DNA into the maternal circulation and/or reduced clearance of circulating DNA from maternal blood *(7)*. The latter mechanism was proposed because the kidney and liver have been suggested to be the main organs for the removal of circulating DNA *(9, 10)*. Because pathologic changes involving the kidney and liver are well described in preeclampsia, it is likely that these processes might reduce the organs' ability to remove DNA from the circulation. However, this mechanism is probably less likely to be important in the current study cohort, who had no renal or liver function abnormalities before the onset of preeclampsia.

It is therefore reasonable to suggest that increased liberation of fetal DNA into the maternal circulation is likely to be the main reason for the early increase in plasma fetal DNA concentrations in subjects who subsequently develop preeclampsia. Theoretically, increased fetal DNA liberation could be secondary to increased entry of fetal cells, such as trophoblasts *(11, 12)* and erythroblasts *(8)*, into the maternal circulation. In support of this possibility is the recent demonstration of increased fetal erythroblast trafficking into the circulation of women who subsequently develop preeclampsia *(13)*. Alternatively, fetal DNA could be liberated directly from dying cells in the placenta. In this regard, widespread apoptosis

has been demonstrated in cytotrophoblasts obtained from the placental beds of preeclamptic pregnancies *(14)*. Increased incidence of apoptosis involving syncytiotrophoblasts has also been reported in preeclampsia *(15)*. Further research on the correlation between fetal DNA concentrations and incidence of placental apoptosis may help to confirm this link.

The potential clinical implication of our findings is that maternal plasma fetal DNA might be used as marker for predicting preeclampsia. However, our data showed that there was overlap in the fetal DNA concentrations between the preeclamptic and control groups. This implies that a relatively low sensitivity and specificity would result if maternal plasma fetal DNA measurement is used as the sole predictor for preeclampsia. Thus, ROC curve analysis (MedCalc 5.0) revealed that the best discrimination between the preeclamptic and control groups was obtained at a fetal DNA concentration of 33.5 genome-equivalents/mL. The sensitivity and specificity at this cutoff concentration were 67% (95% confidence interval, 41–87%) and 82% (95% confidence interval, 65–93%), respectively. The area under the ROC curve was 0.778 (SE = 0.073; 95% confidence interval, 0.639–0.882). Nonetheless, our data open up the possibility of predicting preeclampsia using maternal plasma fetal DNA, especially when used with other biochemical markers such as corticotropin-releasing hormone, α-fetoprotein, inhibin A, and activin A *(16, 17)*. In this regard, it is also important to explore the use of fetal DNA markers outside the Y chromosome so that this type of analysis can be extended to pregnant women carrying female fetuses. Assays that are potentially applicable in this capacity have recently been described *(18–20)*. Finally, our preliminary data would serve to stimulate further large-scale studies to explore the possible correlation of this new marker to the severity of the disease.

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Effects of Repeated Freeze-Thaw Cycles on Concentrations of Cholesterol, Micronutrients, and Hormones in Human Plasma and Serum, *George W. Comstock,[1*] Alyce E. Burke,[1] Edward P. Norkus,[2] Gary B. Gordon,[3] Sandra C. Hoffman,[1] and Kathy J. Helzlsouer[1]* ([1] Department of Epidemiology, Johns Hopkins School of Public Health, Hagerstown, MD 21742; [2] Department of Medical Research, Our Lady of Mercy Medical Center, Bronx, NY 10470; [3] Searle, Skokie, IL 60077; * address correspondence to this author at: Johns Hopkins Training Center for Public Health Research, 1302 Pennsylvania Ave., Hagerstown, MD 21742-3197; fax 301-797-3669, e-mail gcomstock@mindspring.com)

The effects of repeated freeze-thaw cycles on concentrations of various analytes in plasma or serum were of little interest until the growth of plasma and serum banks during the latter part of the 20th century. By 1996, the number of such banks used primarily for cancer research had grown to 115 *(1)*. Many also exist for other purposes, such as the WHO Serum Reference Banks *(2)* and banks associated with cardiovascular studies such as the Multiple Risk Factor Intervention Trial (MRFIT) and the Atherosclerosis Risk in Communities (ARIC) Study *(3, 4)*. Although the need for repeated freezing and thawing of samples can be minimized by storing banked specimens in several small containers *(5)*, it often is necessary to use plasma or serum that has already undergone one or more freeze-thaw cycles. When this occurs, reviewers of re-